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BIOLOGIA MOLECOLARE, CELLULARE E AMBIENTALE

XXX CICLO

Role of HR- and NHEJ-deficiency on *in vivo* abscopal oncogenic response

Ruolo dell'assenza dei meccanismi di riparazione delle DSBs (HR e NHEJ) nella risposta oncogenica abscopale

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A noi tre

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SUMMARY OF THE PROJECT

My PhD project is an *in vivo* radiobiological study focused on understanding how DNA repair mechanisms influence the abscopal damages induced by ionizing radiation.

The central dogma of radiation biology, that biological effects of ionizing radiation (IR) are a direct consequence of DNA damage occurring in irradiated cells, has been challenged by observations that genetic/epigenetic changes occur in unexposed "bystander cells" neighboring directly-hit cells, due to cell-to-cell communication or soluble factors released by irradiated cells.

Cellular effects of IR are of great variety and level, but they are mainly damaging since radiation can perturb all important components of the cell, from the membrane to the nucleus, due to alteration of different biological molecules ranging from lipids to protein or DNA. DNA double-strand breaks (DSBs) are considered the most biologically damaging lesions produced by IR. Notably, DSBs were detected both in directly-exposed and shielded tissues, demonstrating that an incorrect repair of the damage may be responsible for the onset of tumors.

The two main mechanisms by which mammalian cells repair DSBs are Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ).

Using suitable animal models it was possible to identify the DNA-repair system critical for *in vivo* abscopal oncogenesis and hypothesize a pharmacological approach able to modulate the efficiency of the DNA-repair response.

These findings have potential implications in the clinical field and may be crucial for the protection of the normal tissues during medical exposures, or for the potentiation of the radiotherapy outcomes in tumors.

RIASSUNTO

Il mio progetto di dottorato è uno studio di radiobiologia condotto *in vivo* e focalizzato alla comprensione dei meccanismi di riparazione del danno abscopale indotto dall'esposizione alle radiazioni ionizzanti.

Secondo il dogma centrale della radiobiologia, gli effetti biologici delle radiazioni ionizzanti (IR) sono una conseguenza diretta del danno a carico del DNA, che si verifica in cellule direttamente esposte alle radiazioni. Nell' ultimo decennio, numerosi studi sperimentali hanno dimostrato che le radiazioni sono in grado di indurre effetti biologici rilevanti, cambiamenti genetici/epigenetici, anche in cellule non direttamente attraversate dall'energia radiante. La trasmissione indiretta del danno sembra essere mediata dalle comunicazioni intercellulari o dal rilascio di fattori solubili rilasciati dalle cellule irraggiate.

Le radiazioni ionizzanti interagendo con la materia vivente sono in grado di danneggiare in maniera temporanea o permanente le funzioni delle cellule stesse e possono perturbarne tutti i componenti più importanti, dalla membrana al nucleo, a causa di alterazioni di diverse molecole biologiche che vanno dai lipidi alla proteina o al DNA.

I danni più gravi derivano dall'interazione delle radiazioni ionizzanti con il DNA e le rotture a doppio filamento del DNA (DSBs) sono considerate le lesioni biologicamente più dannose.

La presenza di DSBs, come espressione di un danno genetico con potenziale oncogenico, è stata rilevata sia nei tessuti direttamente esposti alle radiazioni sia nei tessuti opportunamente schermati, dimostrando che una riparazione non corretta di tale danno,

potrebbe essere responsabile dell'insorgenza dei tumori in tessuti non direttamante attraversati dall'energia radiante.

I due meccanismi principali che consentono di riparare le DSBs sono la ricombinazione omologa (HR) e la ricombinazione non-omologa (NHEJ).

Usando specifici modelli animali, è stato possibile identificare il meccanismo di riparazione maggiormente coinvolto nell'oncogenesi abscopale *in vivo* e ipotizzare un approccio farmacologico in grado di modulare l'efficienza della risposta di riparazione del DNA.

INTRODUCTION

BIOLOGICAL EFFECT OF IONIZING RADIATION

Ionizing radiation (IR) is a well-known genotoxic agent and human carcinogen that causes different short and long term effects, such as cell death, chromosomal aberrations, DNA damage, mutagenesis and carcinogenesis [(1)].

Cellular effects of IR are of great variety and level, but they are mainly damaging since radiation can perturb all important components of the cell, from the membrane to the nucleus, due to alteration of different biological molecules ranging from lipids to protein or DNA.

Regarding DNA, being the depository for the genetic information in each living cell, its integrity and stability are much more important than other cellular processes and can compromise the viability of the cell. Specific DNA lesion can also induce mutation that cause cancer or other disease as well as contribute to the aging process.

For these reasons, the cells have evolved a network of DNA repair mechanisms to remove different types of DNA damage.

The most deleterious and biologically hazardous forms of DNA damage are represented by double-strand breaks (DSBs), activating cell death responses if unrepaired and promoting genome instability, such as translocations, if misrepaired[(2)(3)]. For instance, a single unrepaired DSB is often sufficient to cause cell death. In addition, inaccurate repair can lead to deletions or chromosomal aberrations, events associated with cancer development and/or genomic instability. Thus, the repair of DSBs is both critical for cell survival and maintenance of genome integrity[(4)(5)]. Cells initiate a highly coordinated cascade of events—collectively known as the DNA damage response (DDR)—that senses the DNA damage, signals its presence, and mediates its repair.

NEW PARADIGM IN RADIOBIOLOGY

The radiobiology of the last two decades has been confronted with phenomena not directly attributable to DNA radiation damage that led to reconsidering the classic paradigm of radiobiology and abandoning the DNA-centric concept of biological damage induced by ionizing radiation[6].

For a long time it was generally accepted that effects of ionizing radiation result from direct ionization of cell structures, particularly DNA, or from indirect damage through reactive oxygen species produced by radiolysis of water, and these biological effects were attributed to irreparable or misrepaired DNA damage in cells directly hit by radiation. Evidence now shows that, as well as these direct DNA damage-dependent effects, irradiated cells also send signals to their neighbours. These non-irradiated cells respond to signals produced by neighbouring irradiated cells by what has been termed a bystander effect.

Bystander effects describe the effects of extracellular mediators from irradiated cells on neighboring non-irradiated cells resulting in radiation-induced effects in unirradiated cells. A simple definition of a radiation-induced bystander response is one in which 'a cell that responds to the fact that its neighbors have been irradiated'[7]. Although the underlying mechanisms are largely unknown, it is widely recognized that two types of cellular communication (gap junctions and/or release of molecular messengers into the extracellular environment) play an essential role.

In contrast to direct irradiation effects, the key characteristic of bystander responses is the dose–response relationships (Fig. 1). The bystander-effect model, postulates that low-dose radiation may be even more damaging than that predicted by the linear no-threshold

model. It has been reported that 1% of cells in cell cultures directly irradiated with an α-particle, "transmitted" the chromosomal damage to 30% of the total cell population[8], via cell-to-cell communication.

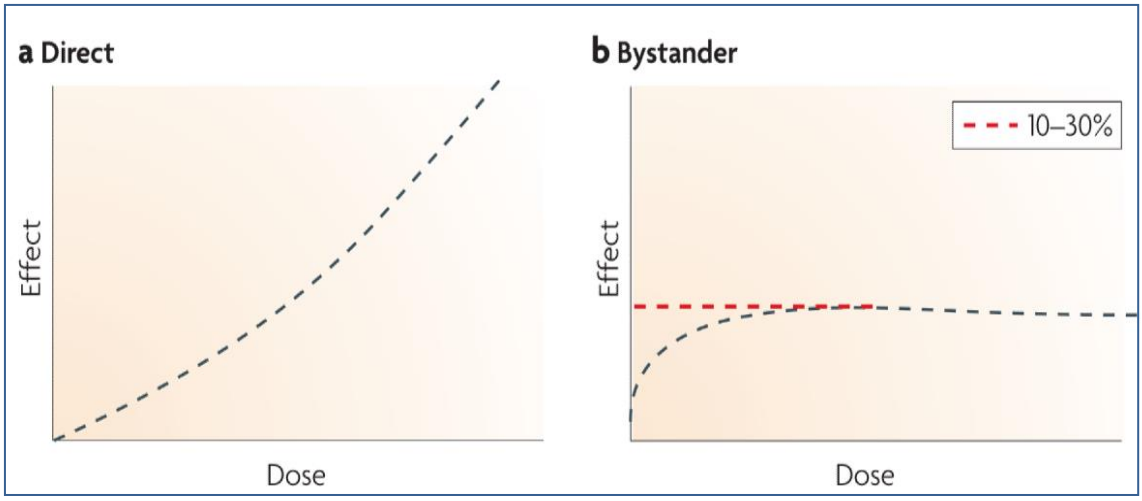

Figure 1. Key aspects of radiation-induced bystander responses. Typical dose response curves for (a) direct and (b) bystander responses.

Some of the common bystander effects or biological end points are evidenced after low-dose irradiation: chromosomal instability, cell killing and delayed cell death, mutagenesis, micronucleus formation, gene and protein expression changes. There is considerable evidence that ionizing radiation affects cells located near the site of irradiation, which respond individually and collectively as part of a large interconnected web[9].

Radiation-induced bystander responses have been observed in a range of cell types, tissue models and *in vivo*. Although the majority of the evidence for bystander effects has come from cellular studies, a range of other responses have been classified as bystander effects in the literature. In humans, in response to radiotherapy, longer-range effects occurring within or between tissues have also been reported and have been termed abscopal, out-of-field or distant bystander responses. Infact the abscopal effect can take place in cells located much further away from the radiation field[10]. Furthermore, the bystander effect is

better understood as the radiobiological events arising from the radiation effect while the abscopal effect refers to clinical changes related to radiation effect. These clinically observed effects appear within a patient's body, sometimes at significant distances from the irradiated tumour, and may be mediated by factors released by irradiated tumour cells and also by cells of the immune system[11].

ABSCOPAL EFFECTS

The term 'abscopal' was defined by Mole in 1953 as a tumor event occurring ''at a distance from the irradiated volume but within the same organism''[12]. The etymology of the word is Latin, with the prefix 'ab' denoting ''position away from'' and 'scopus' as a target for shooting at. Mole posed fundamental questions such as ''How much of this abscopal effect occurs and how is it produced?''. Over 50 years later the answers to these questions remain to be fully elucidated[13]. A broader definition of the term 'abscopal' was given by Andrews as ''...local irradiation of one tissue involved in a response in another or similar tissue remote from the irradiated site''[13], a concept that encompasses both distant tumor and distant normal tissue effects.

Compared with *in vitro* systems, the production of cell-cell transmissible radiation effects can be substantially different *in vivo*, with physiologic cellular connections within tissues, and/or cross talk among tissues/systems allowing long-range transmittal of bystander signals. The search of the effect *in vivo* in mammalian systems represents a priority in the study of cancer risk from low-dose radiation, not only for environmental and occupational exposures but for clinically relevant dose and dose distributions at tissue and whole-body level.

Recently it was confirmed tumor induction in mouse shielded tissues[14], providing the first proof-of-principle that non-targeted (abscopal) effects are factual *in vivo* events with carcinogenic potential. For this study, a knockout mouse model with germline heterozygous inactivation of the oncosoppressor gene Patched (*Ptch1*) was used.

AT A GLANCE[7]

- Radiation-induced bystander responses are defined as the response of cells to their neighbours being irradiated. These have been observed in a range of cell types and measured for a range of end points.
- Long-range, abscopal (out-of-field) effects have also been observed after the clinical use of radiation.
- The main mechanisms involve direct cell–cell communication by gap junction intercellular communication and release of factors into the medium.
- Bystander signalling has a key role in increasing the effectiveness of gene therapy approaches in which common mechanisms involving cytokine signalling and the production of reactive oxygen and nitrogen species have been used to maximize effectiveness.
- With the development of suitable strategies, radiation-induced bystander responses may be used to enhance tumor cell kill or protect normal tissues from the damaging consequences of radiation exposure.

DNA REPAIR PATHWAYS AND MECHANISMS

The two main mechanisms by which mammalian cells repair DSBs are homologous recombination (HR) and Non-Homologous End-Joining (NHEJ) (Fig. 2). These two repair systems differ in their requirement for a homologous template DNA and in the fidelity of DSB repair. HR-directed repair is largely an error-free mechanism as it utilizes the genetic information contained in the undamaged sister chromatid as a template. In contrast, NHEJ is normally error-prone and involves elimination of DSBs by direct ligation of the broken ends. NHEJ is reasoned to be the predominant pathway in mammalian cells operating in all phases of the cell cycle and independent of cell cycle, while HR is restricted to the late-S and G2 phases. Several human diseases have been reported to derive from deficiencies in HR or NHEJ, and these exhibit neurological, immunological and developmental defects, as well as radiation sensitivity, premature aging phenotypes and cancer predisposition[15][16][17][18][19][20].

HOMOLOGOUS RECOMBINATION (HR)

HR resolves DSBs during the S and G2 phases of the cell cycle. The pathway appears to have mainly evolved to cope with the one ended DSBs that are formed upon replication fork collapse, most commonly at a polymerase blocking lesion, during duplication of chromosomal DNA in dividing cells. The repair mechanism is pivotal to maintain replication fidelity and employs an intact sister chromatid as a template for information exchange and faithful repair. HR has been proposed to be initiated by recognition of the DSB by the MRN complex, which is comprised of the MRE11, RAD50,

and Nijmegen breakage syndrome 1 (NBS1) proteins[21]. This complex acts as a break sensor and recruits the protein kinase, ataxia telangiectasia mutated (ATM), to DSB sites, facilitating the subsequent steps of the recombination process[22][23][24].

NON HOMOLOGOUS END JOINING (NHEJ)

NHEJ is the major DSBR system in higher eukaryotes[25], particularly during phases of the cell cycle when a homologous sister chromatid is absent. NHEJ proteins are also involved in introducing antibody diversity via V(D)J recombination[26]. Some reports have recently described how NHEJ contributes to the maintenance of telomere integrity as well[26][27][28][29][30]. NHEJ entails three main steps, which ultimately culminate in the direct ligation of two DNA ends in close spatial proximity: i) recognition of the two-ended DSB, ii) processing to remove non-ligatable termini or other forms of DNA damage at the break and to reveal short stretches of microhomology, and iii) joining of two suitable ends. In general, there is competition between the recognition complexes of HR and NHEJ for DSB termini, with pathway selection mostly being influenced by the stage of the cell cycle. While NHEJ can operate during all phases of the cell cycle, it is most active during G1[31][32]. Due to the end processing step, NHEJ often results in an error-prone outcome, with partial loss of genome information at the site of the DSB.

To initiate NHEJ, the Ku70/Ku80 (Ku) heterodimer binds directly to the two DSB ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). This multiprotein complex both stabilizes and aligns the DNA ends[33][34][35]. The interaction between two DNA-PKcs positioned at each DSB terminus activates its intrinsic protein kinase activity, leading to DNA-PKcs autophosphorylation and dissociation. Depending

on the complexity of the DSB and the nature of the ends, different processing factors are then recruited.

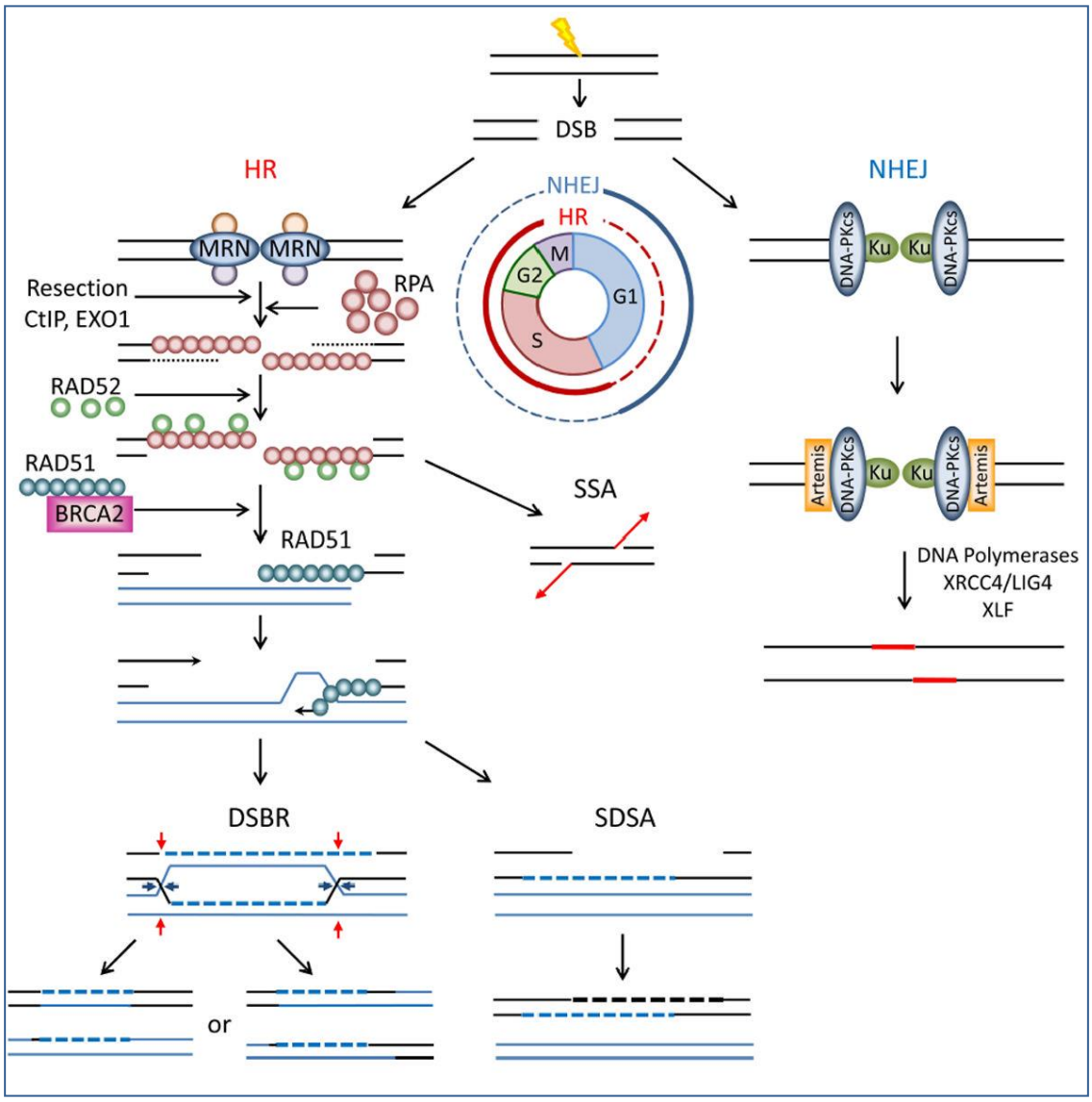

Figure 2. DSBR is divided into two major pathways: HR and NHEJ. HR operates in dividing cells and in S phase, whereas NHEJ can function in both dividing and non-dividing cells and independent of cell cycle[36].

ANIMAL STUDIES

Animal studies play an important role in improving the understanding of radiation carcinogenesis. The use of tumor data from animal studies is needed as a complement of epidemiological studies of human populations to develop estimates of radiation cancer risk at low doses. In addition, animal experiments provide valuable insights into the mechanisms of radiation interaction with living cells and organisms, allowing clarification of the pathways of tumorigenesis, and of the factors modifying radiation risks. Animal studies, however, are hampered by the requirement of very large animal numbers to reach statistical significance, particularly at low radiation doses. Thus, genetically manipulated, radiation susceptible mouse models represent a powerful tool to help assessment of risk. In addition, a strong need exists for systems that provide information on the mechanisms whereby a "hit" normal cell develops into a tumor.

The animal models used in this study were *Ptch1* heterozygous mice (*Ptch1*$^{+/-}$) and *Rad54* and *DNA-PKcs* null mice (*Rad54*$^{-/-}$ and *DNA-PKcs*$^{-/-}$) and *DNA-PKcs* heterozygous mice (*DNA-PKcs*$^{+/-}$).

PTCH1 KNOCKOUT MICE

Ptch1 heterozygous mice have been used as a reference model in this study. This knockout mouse model presents a germline heterozygous inactivation of the developmental and oncosuppressor gene *Patched* (*Ptch1*), a condition predisposing humans and mice to developmental abnormalities, CNS and other tissue tumors, in addition to radiation susceptibility.

Mice lacking one *Ptch1* allele are generated through disruption of exons 6 and 7 in 129/SV ES cells, and maintained on CD1 background (Fig. 3)[37]. CD1 is an outbred stock, and brother-sister mating was avoided to minimize consanguinity.

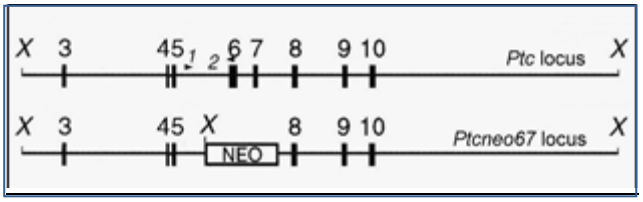

Figure 3. Gene construct used to produce Ptch1$^{neo6\text{-}7/+}$ knockout mice[37].

The *Ptch1* gene is an oncosuppressor and the Ptch1 protein forms part of the complex of Sonic Hedgehog (Shh) signal pathway, involved in many processes that govern embryonic development and cell proliferation. A constitutional defect in one of two copies of *Ptch1* is responsible for Gorlin syndrome, family syndrome that predisposes tumor development (Gorlin, 1995). Gorlin patients inherit a mutated copy of the *Ptch1* gene, a condition that causes many developmental anomalies and predispose to spontaneous tumorigenesis, such as basal cell carcinomas of the skin, rhabdomyosarcomas[38][39], and medulloblastomas (MB)[40]. Gorlin patients also exhibit a strong susceptibility to the effects of ionizing radiation, causing multiple cell basal cell carcinoma in the skin exposed to radiotherapy.

Ptch1 homozygous inactivation is incompatible with life because induces defects in the development of nervous and cardiovascular systems and animals die during gestation. The heterozygous *Ptch1*$^{+/-}$ mice, however, are vital and represent a perfect model of Gorlin syndrome, recapitulating all the typical symptoms, and maintain their susceptibility to tumor development following exposure to ionizing radiation.

This animal model has been extremely useful in identifying early stages in the development of basal cell carcinoma and MB and in deepening to better understand the molecular events responsible for the onset of these cancers[41][42]. Moreover, this animal models has been resolutely to dissect the molecular mechanisms controlling the carcinogenic potential of abscopal effect induced by irradiation and represents a paradigm of radiation hypersensitivity leading to cancer.

MEDULLOBLASTOMA DEVELOPMENT IN *PTCH1*$^{+/-}$ MICE

Hemizygous *Ptch1* mice have many features of Gorlin syndrome, including predisposition to MB development.

Medulloblastoma is a primitive neuroectodermal tumor that develops in the cerebellum, and represents the most common brain tumor of childhood[43][44], accounting for about 20% of all brain tumors in infancy[45].

Although the cell of origin is still unknown, MB is believed to arise from the cerebellar granule neuron precursors (CGNPs), which undergo massive proliferation and migration in response to Shh secreted by Purkinje neurons immediately after birth[46][47]. The Shh pathway is crucial for normal development of the cerebellum, because it governs the proliferation of CGNP cells. It has been suggested that MB arises as an aberration of normal developmental processes: a CGNP cell fails to exit the cell cycle at the appropriate time and remains in the external granule/germinal layer (EGL), eventually expanding to form a tumor[48]. The EGL persists, until postnatal day 21 (P21) in mice and into the second year of life in humans. As the cerebellum develops, CGNPs forming the EGL undergo a period of rapid and massive clonal expansion with a peak at P5–7 in the mouse before

migrating inward, across the Purkinje cell layer, to eventually form the post-mitotic neurons of the internal granule layer (IGL) (Fig. 4).

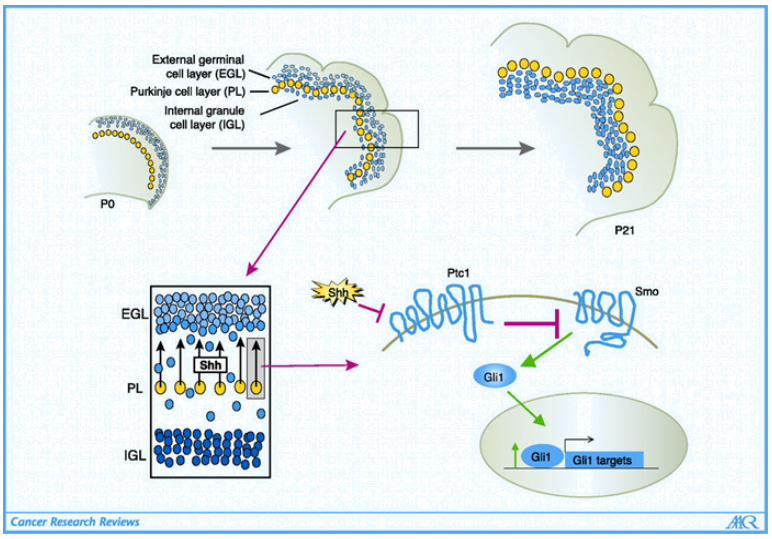

Figure 4. Shh pathway is critical for the normal development of the cerebellum. Shh released from the Purkinje cells acts on overlying GNP cells in the EGL, leading to proliferation. After this period of Shh-dependent proliferation, granule neurons exit the cell cycle, begin to differentiate, and migrate inwards, past the Purkinje cell layer to reside in the IGL in the mature cerebellum[49].

The *Ptch1*$^{+/-}$ mice develop MB spontaneously with an incidence of 7%, but the exposure to a single dose of ionizing radiation during the first week of postnatal life, increases significantly this incidence.

The maximum incidence of this tumor (81%) is observed when the irradiation is performed in mice *Ptch1*$^{+/-}$ at 1 day of postnatal age. DNA damage induced by radiation strongly promotes MB development in newborn *Ptch1*$^{+/-}$ mice in which the EGL is still proliferating[50]. The period of sensitivity to induction of MB by ionizing radiation is therefore strictly limited to early days of post-natal life, indicating that at the time of irradiation, target cells are still in a receptive phase for radiation induced stochastic effects that can result in enhanced tumorigenesis[51].

Utilizing a mouse model of radiosensitivity, the *Ptch1* heterozygous mice, it was established the first proof-of-principle that bystander/abscopal effects are factual *in vivo* events with carcinogenic potential[14]. In particular, monitoring genetic damage and MB induction in shielded cerebella of *Ptch1* mutant mice after X-ray exposure of the remainder of the body, we demonstrated that bystander/abscopal signals can initiate tumorigenesis in unexposed CNS *in vivo*; more recently, we investigated the mechanisms involved in the transmission of long-range bystander/abscopal responses, showing a key role of gap junction intercellular communications (GJICs) in propagating radiation stress signals *in vivo*[52]. When *Ptch1*$^{+/-}$ mice were partial-body irradiated in neonatal age with 3 Gy of X rays, using individual lead shields for protection of mouse heads, is possible to detect DNA double-strand breaks and apoptotic cell death in shielded cerebellum *in vivo*. Associated with these genetic events, there was a remarkably increased cerebellum tumor (i.e., medulloblastoma) rate compared with controls (39% vs. 7%) in *Ptch1*$^{+/-}$ mice in which only the body, but not the head had been irradiated. Remarkably, the short-term cellular responses were not specific of radiosensitive *Ptch1*$^{+/-}$ mice, as wild-type siblings showed identical abscopal phenomena in neural precursors of P2 cerebellum. Therefore, these effects are not restricted to atypical or limited experimental models, rather they are potentially reproducible in any mouse strain.

A further important feature of the both control and irradiated cerebella of *Ptch1*$^{+/-}$ mice is the presence of abnormal cerebellar EGL regions ranging from small areas of hyperproliferation of granule neurons to overt nodules.

In unirradiated *Ptch1*$^{+/-}$ mice these lesions were evident in 3/5 (60%) mice at P21 and 3/5 (60%) at P31. However, only 7% of unirradiated mice developed MB, indicating that only a subset of hyperproliferating areas progress to MB. Most of these spontaneous lesions will regress by adulthood, since they are not detected in mice autopsied at later times.

For this characteristics in *Ptch1*$^{+/-}$ mice the development of MB occur in a multistage process by early-onset microscopically recognizable preneoplastic lesions (PNLs), with microscopic dimensions, to full-blown tumor.

The progression of MB PNLs is a well characterized process, developed by ENEA lab, which allows for the investigation of the early genetic events and tumor incidence in a shorter time[53].

The microlesions in the EGL can have variable dimensions and show different degrees of morphological alteration and can be classified as: hyperproliferation areas, that are small in size and exhibit slightly impaired histological features; larger nodular lesions, with a high component of atypical cells and lesions similar to blown medulloblastoma, but smaller and still circumscribed (Fig. 5).

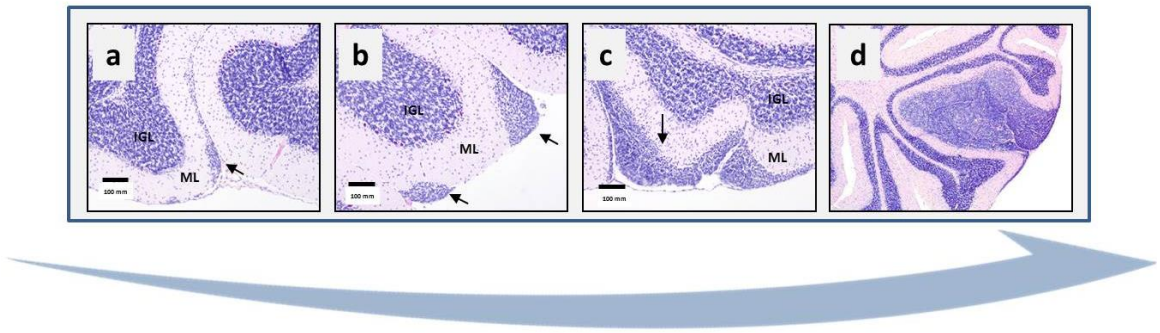

Figure 5. Classification of hyperplastic cerebellar lesions according to criteria of increasing degree of altered cellular morphology and size: (a) hyperproliferation of EGL, (b) micronodule, (c) nodule, and (d) microtumor.

As previously described[54], progression of MB PNLs occurs in progressive time points from 2 to 8 weeks of age varying from focal subpial aggregates of CGNPs (2 wks) to overt microtumors (8 wks) (Fig. 6).

In this study, PNLs incidence was evaluated in asymptomatic mice euthanized at 8 weeks of age. At this age, microtumors are identified on the outer surface of the cerebellum, and represent bona fide neoplasms closely predicting final brain tumor incidence.

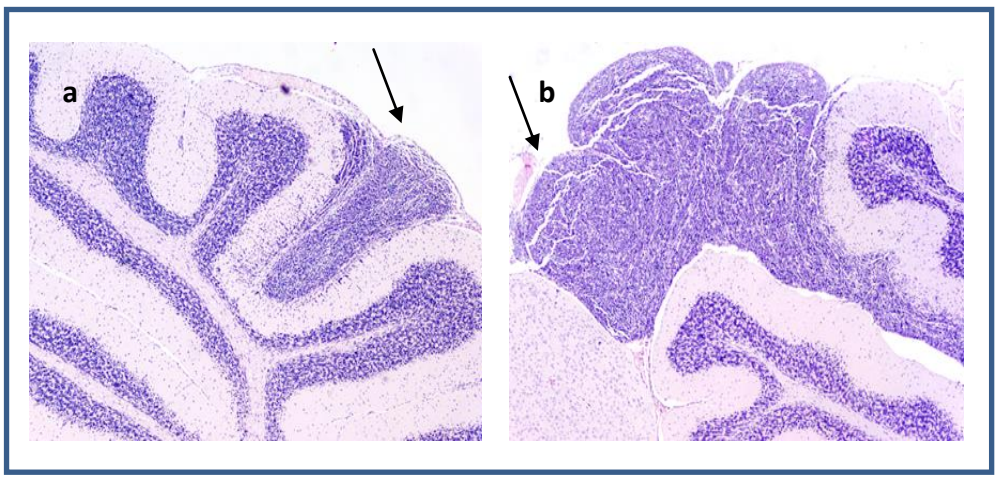

Figure 6. PNLs occurs in progressive time points from 2 to 8 weeks of age varying from (a) focal subpial aggregates of CGNPs (2 wks) to (b) overt microtumors (8 wks).

The stage of preneoplastic lesions in the cerebellum are classified according to histological and dimensional analysis as follows: hyperproliferation area (area < 5×10^5 μm^2) and asymptomatic microtumor (area > 5×10^5 μm^2). The PNLs incidence will be expressed as the percentage of mice with abnormal cerebellar regions with morphology varying from focal subpial aggregates of GCPs to overt nodules or microtumors.

DNA-PKcs AND *RAD54* KNOCKOUT MICE

DNA repair is critical for neural development, and defects in this process underlie neurological disease. Many human syndromes with DNA repair deficiency are, in fact, characterized by neuropathology, such as neurodegeneration, microcephaly or brain tumors, suggesting that responding to DNA DSBs is essential for neural homeostasis[55]. Given the importance of *Ptch1* for CNS development and tumorigenesis, I sought to determine the potential relationship with DNA repair pathways. Although mouse studies are crucial for the analysis of cancer frequency, they are usually less informative for the dissection of end points, such as DSB processing and cell survival. The mouse cerebellum, however, is characterized by extended postnatal development, and the effects of inefficient HR or NHEJ on the processing of DNA damage can be detected and quantified *in vivo.*

By literature emerge that knocking out genes in mice has facilitated the identification of DNA repair genes critical for oncogenesis. In particular, mechanisms of DNA repair can be manipulated precisely to create *in vivo* models whereby the underlying processes of tumorigenicity are accelerated or attenuated, depending on the composite alleles carried by the mouse model. Recent evidence indicates that brain tumors may be linked to defects in DNA-damage repair processes, as various combinations of targeted deletions in genes

controlling cell-cycle checkpoints, apoptosis and DNA repair result in MB in mice. For examples, such models have evolved to study MB development inactivating of *Ligase IV*, *Xrcc2*, *Brca2* and *PARP-1* together with targeted deletion of *p53*[56][57][58][59].

In keeping with this, to test the role of defective HR or NHEJ in abscopal tumorigenesis in CNS *in vivo*, the aim of this work is to place on a lifetime study, control and irradiated *Ptch1*$^{+/-}$ mice with no functional *Rad54* or *DNA-PKcs* alleles or with one functional *DNA–PKcs* alleles, in order to evaluate abscopal MB development.

Ptch1$^{+/-}$ mice, maintained on CD1 background, and *Rad54* and *DNA-PKcs* null mice, both maintained on C57BL/6 background, are available at the ENEA animal facility.

GENETIC BACKGROUND: CD1 *VS* C57BL/6

The genetic background can have a significant effect on mutant phenotype and, in particular, may cause individual variability in biological responses to radiation. The "genetic background" (in which each gene functions) is defined as the genotype of all other related genes that may interact with the gene of interest, and therefore potentially influences the specific phenotype.

C57BL/6 is an inbred strain that present refractory to many tumors and it is classified as the most commonly radiation-resistant mouse strain.

In *Ptch1* heterozygotes, variable incidences of spontaneous MB have been reported depending on background[50][60] demonstrating that tumorigenesis is modified by mouse strain-specific alleles interacting with *Ptch1* haploinsufficiency (Fig. 7).

As previously suggested[61], the gene-environment interactions are important determinants

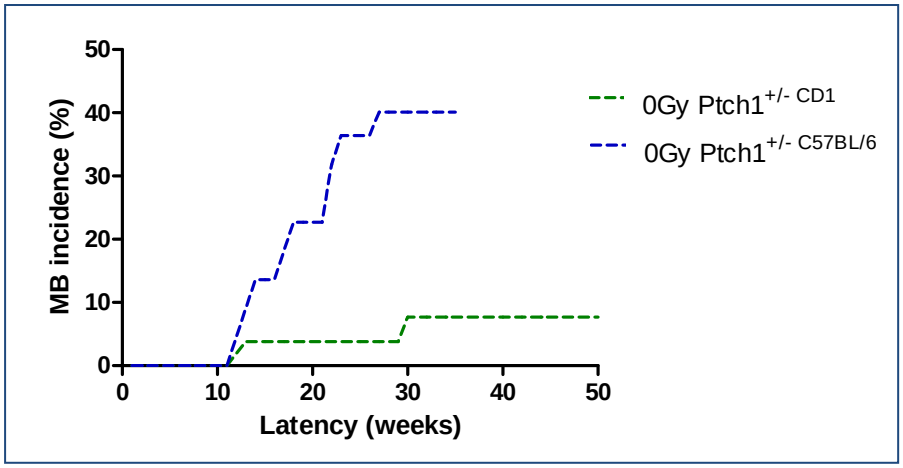

Figure 7. Comparison of spontaneous MB incidences in *Ptch1* mice maintained on CD1 and C57BL7/6 background.

in cancer risks from radiation and modifying the cancer susceptibility also the radiation-induced cancer could be modified.

Whereas heritable factors are potent modifiers of radiation-related cancer risk, the C57BL/6 background is not ideal to study the abscopal tumor response conditioned by inactivation of HR or NHE; for this reason it was necessary to transfer the genetic background of *Rad54* and *DNA-PKcs* mutant mice.

Normally to transfer the genetic background, mice should be backcrossed at least for 10 generations. During my PhD, I completed the genetic background transfer, realizing the last 3 backcrosses. Before starting the experimental design, it was necessary to verify the eligibility of mouse model transferred on CD1 background comparing the spontaneous rate of MB in *Ptch1*$^{+/-\ C57BL/6\rightarrow CD1}$ mice with *Ptch1*$^{+/-\ CD1}$ mice.

A tumor incidence of about 5% is found in both groups, do not different from that obtained previously in *Ptch1*$^{+/-\ CD1}$ mice. This result demonstrate that genetic background was efficiently transferred from C57BL/6 to CD1 (Fig. 8).

Then, F1 generation was intercrossed to produce large F2 populations as illustrated in figure 9.

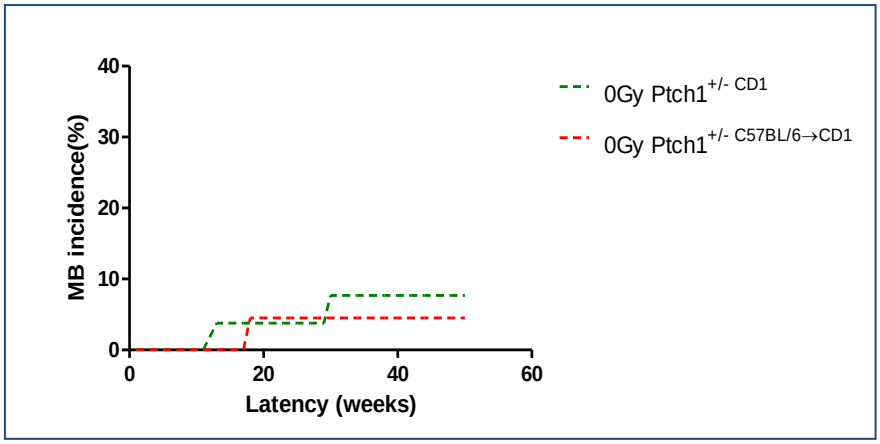

Figure 8. Comparison of spontaneous MB incidences after genetic background transfer. The incidence of about 5% in both groups demonstrates that genetic background was efficiently transferred from C57BL/6 to CD1.

Because only *Ptch1*$^{+/-}$ mice are prone to MB, the effects of *Rad54* and *DNA–PKcs* inactivation on the processing of radiation-induced DSBs, as well as in the molecular pathogenesis of CNS cancer, were evaluated in double knockout mice (*Ptch1*$^{+/-}$*/Rad54*$^{-/-}$, *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$, *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$).

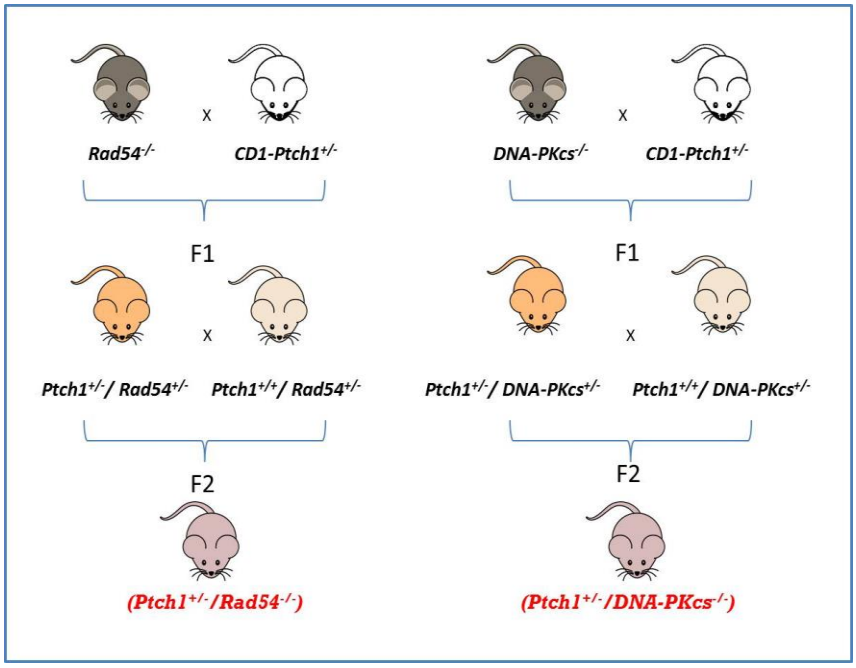

Figure 9. Double knockout mice produced for the experiments.

RATIONALE OF THE PROJECT

The indirect anticancer effect of radiation therapy (RT) on tumor cells outside the irradiation field has been referred to as an abscopal/bystander effect in many human malignancies.

Radiation is a key modality in the treatment of cancer and it is therefore of great practical interest to elucidate the mechanisms of *in vivo* non-targeted effects that can be modulated to enhance cancer cell killing, or lead to improved protection of normal tissues adjacent to cancerous lesions, including reduced risk of second malignancies.

The success or failure of standard clinical radiation treatment is determined by few crucial factors: repair of DNA damage, redistribution of cells in the cell cycle, repopulation, and reoxygenation of hypoxic tumor areas[62].

Concerning DNA damage repair, previous work[63][64] has stressed the importance of DNA-DSB repair in bystander phenomena, and the possibility that unrepaired or misrepaired DSBs underlie bystander induction of chromosomal aberrations and mutations involving large-scale genetic changes.

In particular, several molecules taking part in the DNA repair response, among them DNA-PKs and RAD54, may represent clinically relevant targets for chemical or pharmacological intervention.

With the development of suitable strategies, radiation-induced bystander responses may give new additional opportunities to a more successful clinical approach[65] and to increase the efficacy of therapy approaches.

OBJECTIVES

GENERAL

Elucidate the contribution of the DNA repair system (Homologous Recombination (HR) or Non-Homologous End Joining (NHEJ) pathways), in the resolution of the abscopal DNA damage induced by ionizing radiation.

SPECIFIC

- Generate of specific animal models able to elucidate the contribution of two DSBs repair pathways (non-homologous end joining (NHEJ) and homologous recombination (HR)), in the resolution of the abscopal DNA damage (*Ptch1*$^{+/-}$*/Rad54*$^{-/-}$, *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$).
- Analyze the *in vivo* abscopal oncogenic response occurring in shielded brain target tissue by carcinogenic study and short-term response after irradiation.
- Evaluation of early lesions/microtumors of asymptomatic mice from each experimental group (*Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$) at 8 weeks of age.
- Development of pharmacological strategies to modulate the DNA-DSB repair and tumor response modulating pharmacologically the HR- and NHEJ-DNA repair pathways.

RESULTS

SHORT-TERM ANALYSIS: APOPTOSIS IN SHIELDED-IRRADIATED EGL

To better characterize the short term response in our experimental model, and understand the molecular mechanisms involved, we analyzed the apoptotic levels in CGNPs proliferating postnatally in the EGL of the developing cerebellum, 6h post-irradiation[14].

Comparing the apoptotic response (Fig. 10), in basal condition, we can observe an rising trend in apoptotic levels in *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice respect *Ptch1*$^{+/-}$ mice (*P* value= 0.2771) and a significantly increase in *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ respect *Ptch1*$^{+/-}$ mice (*P* value = 0.0307). This result strengthens the concept that the absence of DNA repair pathway, in and of itself, influences the response of endogenous damage.

After shielded irradiation, comparing *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ and *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice, the apoptotic response is greater (approaching the significance, (*P* value= 0.0993) in *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ mice, assuming that the absence of the NHEJ pathway can plays a critical role in the radiation abscopal response.

Shielded irradiation induces a significantly increase in apoptotic response respect to control mice in all genotype, confirming the presence of radiation-bystander component of damage to shielded brains.

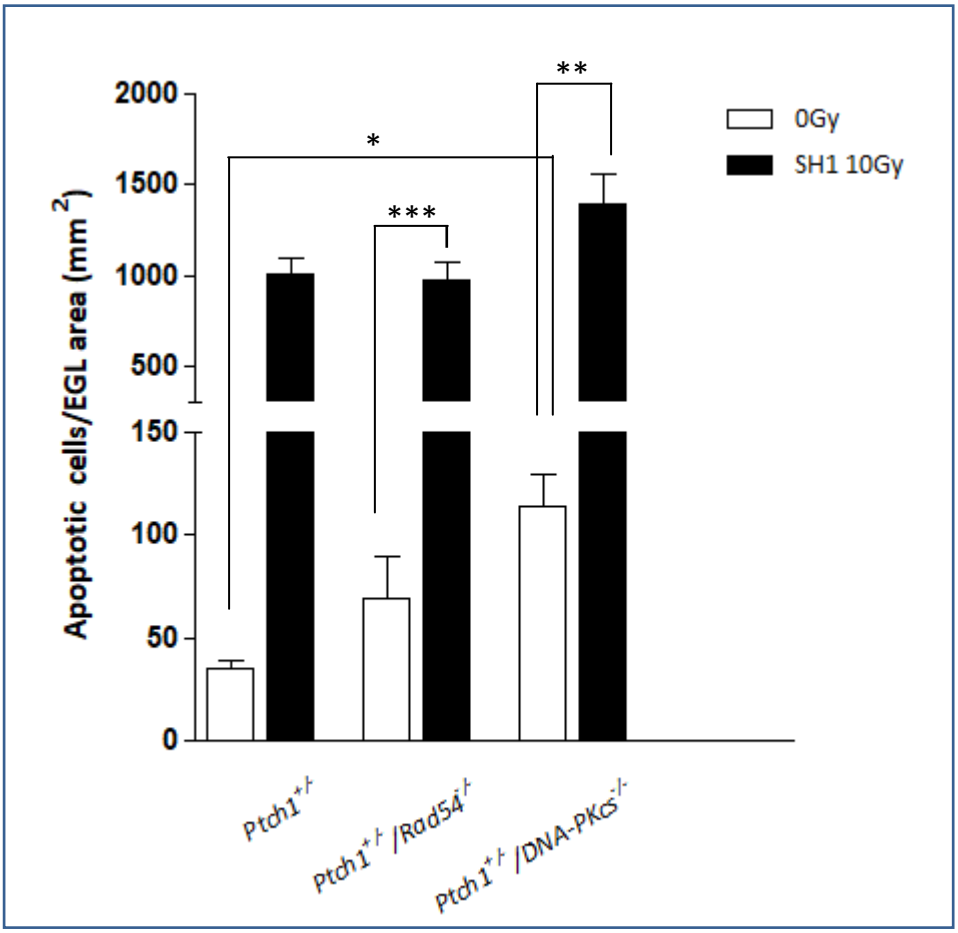

Figure 10. Apoptotic index in basal condition and after shielded irradiation between *Ptch1*$^{+/-}$, *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$mice. (**P* value=0.0307; ***P* value=0.0993; Student's t-test).

MEDULLOBLASTOMA INCIDENCE IN SHIELDED CEREBELLUM OF DOUBLE KNOCKOUT MICE

For tumor induction, double knockout mice *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ (n= 36) and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ (n= 41) were collected and shielded-irradiated (SH1) with a single dose of 10 Gy of X-rays at post-natal day 2, the age of peak susceptibility of MB *Ptch1*$^{+/-}$ mice[37][54].

Control mice, *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ (n= 40) and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ (n=41) were left untreated. Mice were placed on a lifetime study and monitored for tumor development.

DNA-PKcs deficient mice have severe combined immunodeficiency due to their V(D)J recombination defect and, regardless of radiation exposure, these mice have a shorter lifespan and show an earlier onset of numerous aging related pathologies than corresponding wild-type littermates. However, when kept in a ventilated rodent housing system, their lifespan increases significantly. Although the shielding geometry adopted in this study is able to preserve the majority of vital organs, minimizing the impact of acute radiation syndrome and related delayed effects, *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ SH1-irradiated mice showed a very high mortality compared with their unirradiated counterparts. I observed that the combination of *DNA-PKcs* deficiency and neonatal irradiation with a high dose of radiation, even though delivered in a shielded way, resulted in a shortened lifespan[66]. Mice exhibit lethality after 2-4 days post irradiation.

This event has invalidated the initial hypothesis and it was necessary to interrogate mice heterozygous for *DNA-Pkcs* (*Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$).

MEDULLOBLASTOMA PRENEOPLASTIC LESIONS

In spite of *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice show a better survival after irradiation, I decided to evaluate the abscopal radiation effects on MB development, through the analysis of microscopically recognizable PNLs at 8 weeks of age.

A new set of animals was recollected. *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice (n= 41) and Pt*ch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ (n= 38) were shielded irradiated and analyzed for PNLs incidence. Control mice, *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ (n= 24) and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ (n=52) were left untreated.

I performed histological examination of cerebella after irradiation with 10 Gy in SH1 settings and they show a PNLs frequency of 31,7% (13/41) in *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice compared with 42.10% (14/38) incidences of *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice (Fig. 11).

The incidence of positive mice for cerebellum abnormalities was similar between control group being: 29.16% in *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice *vs* 25% in *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice.

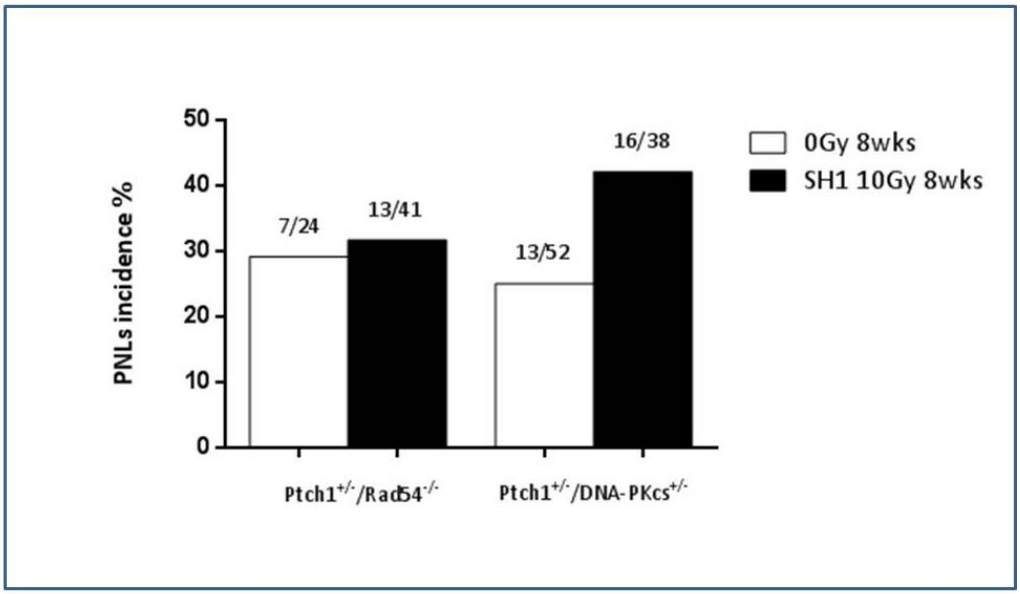

Figure 11. PNLs frequency of control and shielded irradiated *Ptch1*$^{+/-}$*/Rad*$^{-/-}$ and *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice.

To better understand the results about PNLs incidence of the SH1 irradiated groups, I carried out a dimensional analysis on positive mice, classifying PNLs according to histological and dimensional analysis as hyperproliferation area (HA, area < 5×10^5 μm^2) or asymptomatic microtumors (MT, area > 5×10^5 μm^2) (Fig.12a).

After SH1 irradiation, the relative frequency of MT in the *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice (12%) were lower compared with *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice (29%), but the distribution of areas exhibits that the MT of *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice are basically larger compared with

Ptch1$^{+/-}$*/Rad54*$^{-/-}$ mice (P value= 0.6166), suggesting that, in the absence of NHEJ the progression of cerebellum abnormalities was accelerated (Fig. 12b).

Moving attention on HA, because their size is the same between the groups, I decided to carry out the analysis of proliferation and differentiation markers to understand the propensity of HA to develop into medulloblastoma or to regress to normal tissue and to evaluate the influence of DNA repair pathways on their fate.

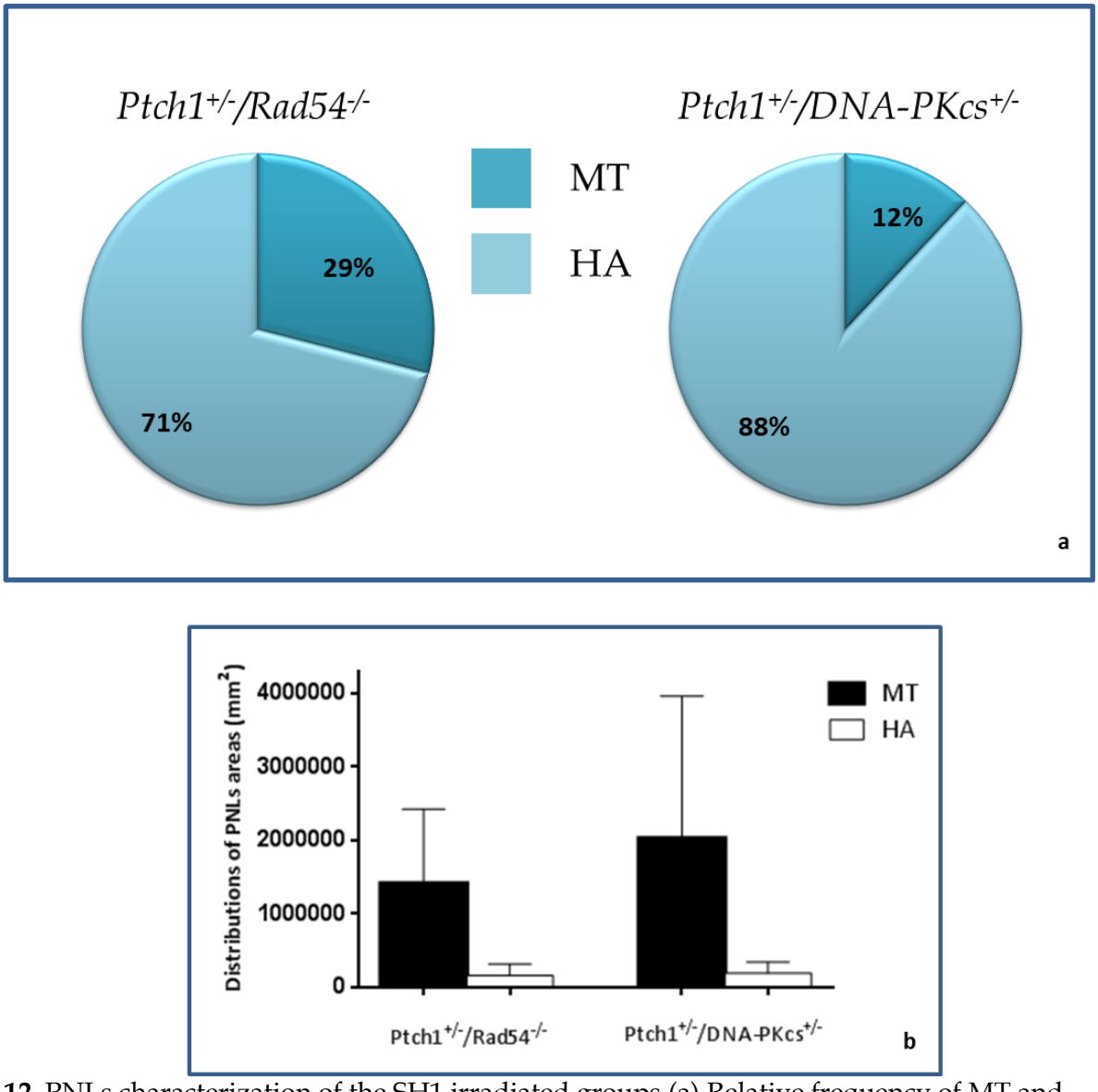

Figure 12. PNLs characterization of the SH1 irradiated groups.(a) Relative frequency of MT and HA in *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ and *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice; (b) Distribution of areas of MT and HA in *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ and *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice.

Serial sections of each lesion were immunostained with an anti-PCNA antibody, a marker of proliferation, and with an antibody against NeuN, a marker of neuronal differentiation (Fig. 13).

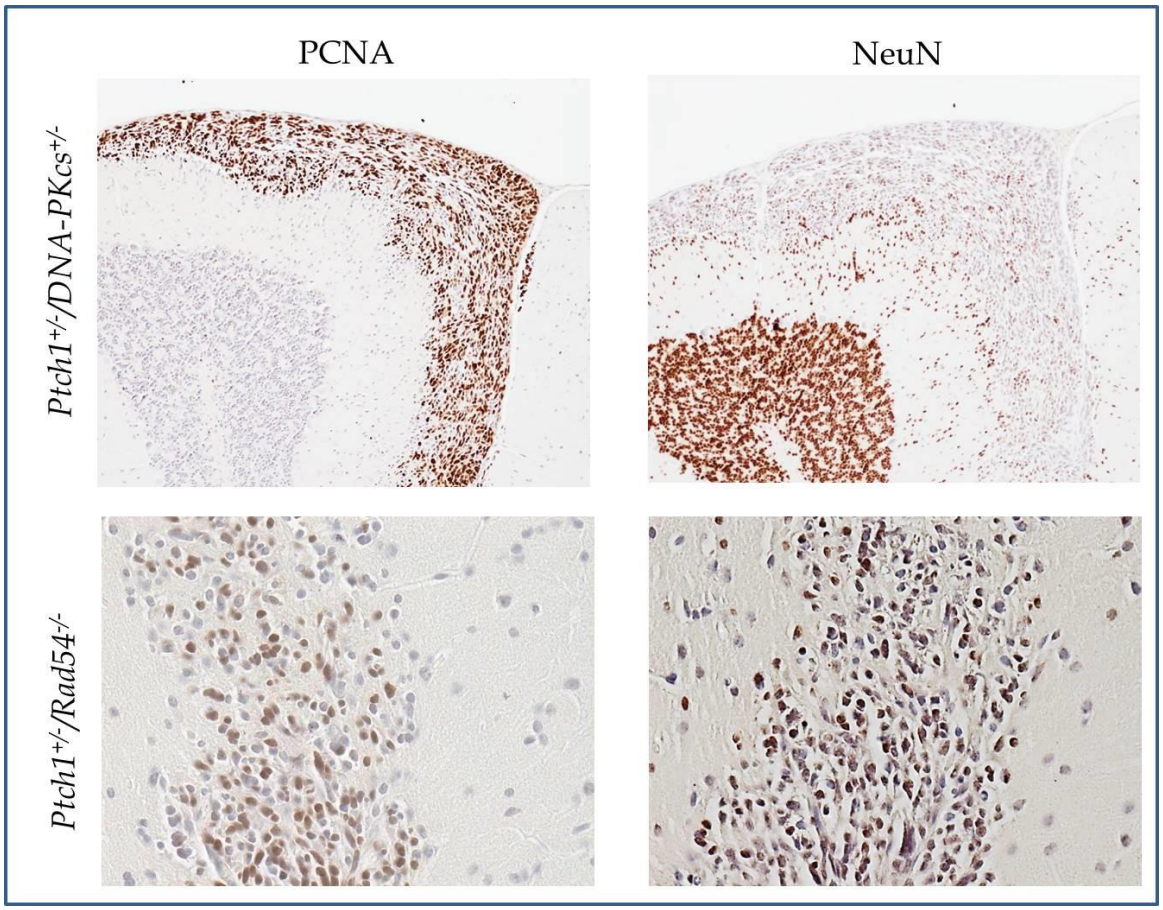

Figure 13. Representative images of PNLs of *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ and *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice immunostained with anti-PCNA and anti-NeuN.

As reported in figure 14, HA of *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice showed 3,2% of PCNA and 0,27% of NeuN-positive cells, showing a significant increase of proliferating cells and a virtual absence of differentiated cells (*P* value= 0.0220) respect *Ptch1*$^{+/-}$*/Rad*$^{-/-}$ mice (0,39% of PCNA and 0,96% of NeuN; *P* value= 0.0288), suggesting a more likely commitment to tumor progression.

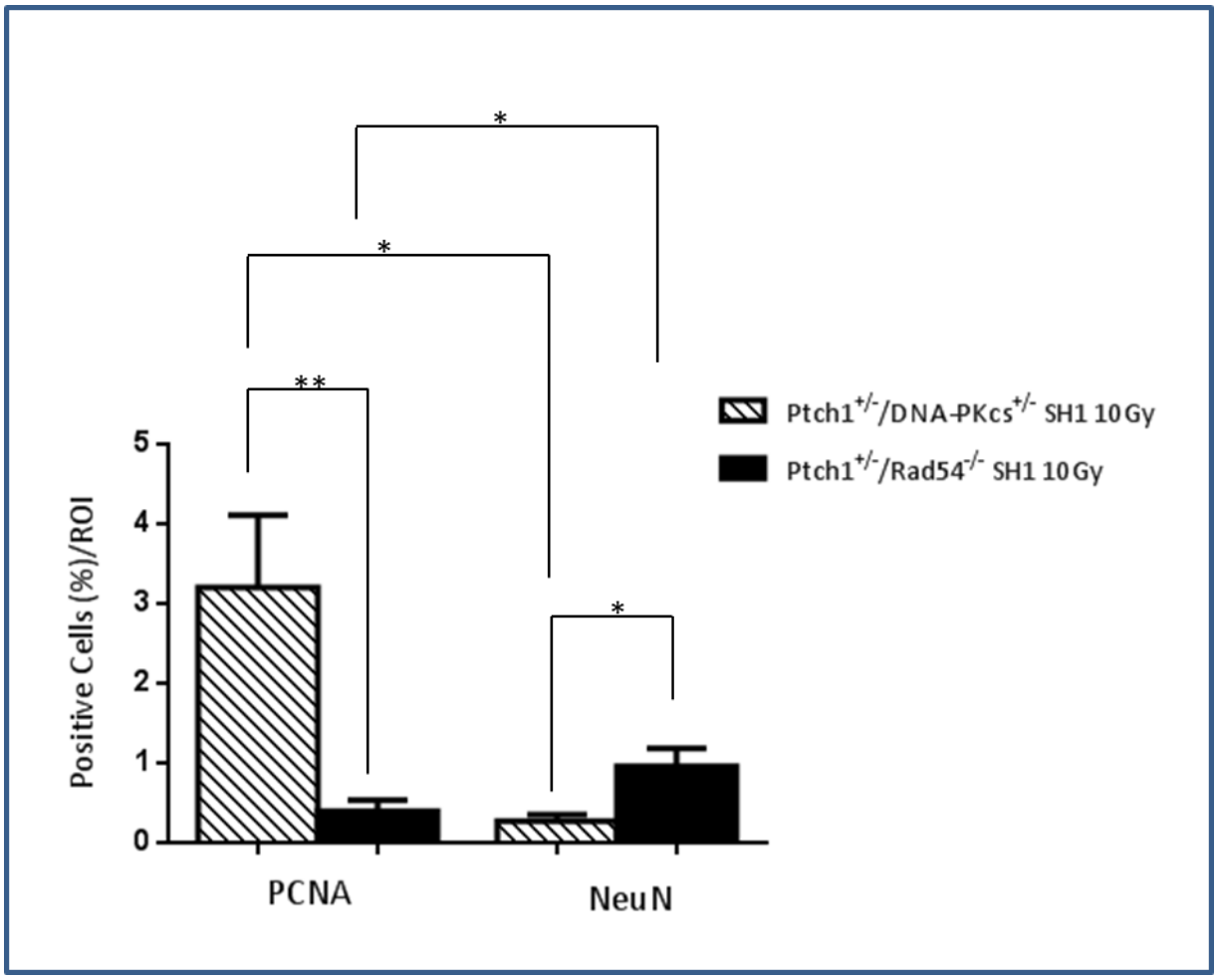

Figure 14. Immunohistochemical analysis showed significantly higher PCNA positive cells along with lower NeuN expression in PNLs from *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ SH1 irradiated mice compared to counterpart (PCNA *vs* NeuN *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice (**P* value= 0.0220); PCNA *vs* NeuN *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$ mice (**P* value= 0.0288; Student's t-test).

If we assume that the HA of *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ mice are intended to become blown medulloblastoma, the final incidence of MT could become statistically significant changing the result beyond the initial observation (Fig. 15).

Therefore, lack of functional *DNA-PKcs* allele significantly increased the rate of radiation-induced tumor development in non-targeted *Ptch1*$^{+/-}$ cerebellum, providing unequivocal evidence for the role of functional *DNA-PKcs* in the expression of oncogenic damage in tissues remote from the irradiated field.

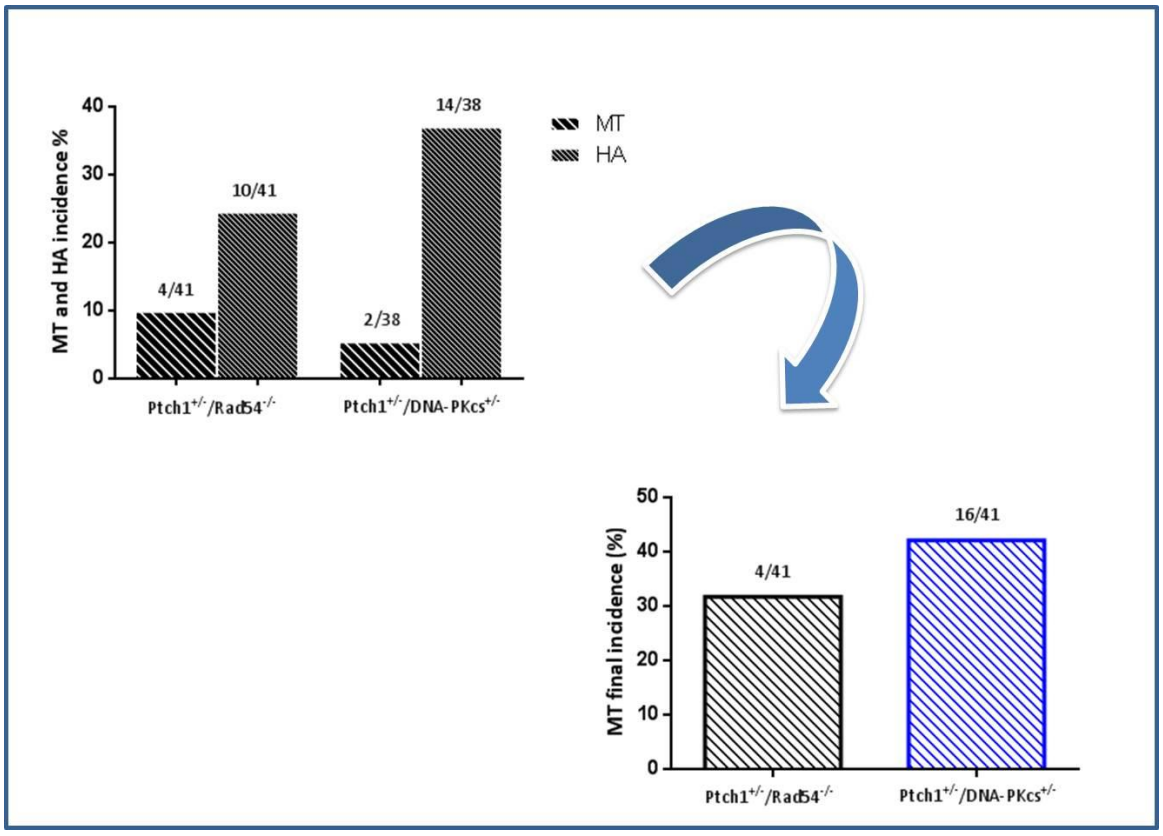

Figure 15. Predicted final incidence of MT in *Ptch1*$^{+/-}$*/DNA-PKcs*$^{+/-}$ and *Ptch1*$^{+/-}$*/Rad*$^{-/-}$ mice.

TPO INCREASES DNA-PKCS DEPENDENT DNA REPAIR EFFICIENCY

Thrombopoietin (TPO) is a protein also known as megakaryocyte growth and development factor. It is a glycoprotein hormone produced by the liver and kidneys which regulates the production of platelets. It stimulates the production and differentiation of megakaryocytes, the bone marrow cells that bud off large numbers of platelets[67]. Recent works has uncovered an unknown function at TPO related to regulation of DNA damage response.

Starting from the results that the absence of NHEJ is critical in the resolution of the abscopal damage and the recent work of De Laval *et al.*, in which it was demonstrated the

new role of TPO in the regulation of NHEJ DNA damage repair efficiency[68][69], I analyzed the TPO's effects after injection *in vivo,* in terms of apoptotic response.

TPO was somministrated 30 min before the SH1-10Gy irradiation in *Ptch1*$^{+/-}$ mice, causing a significant decrease in apoptotic response (Fig. 16) respect untreated irradiated mice. This result shows that TPO can control the DSB repair machinery and that TPO-mediated significantly decrease (*P* value = 0.0017) in DNA damage in *Ptch1*$^{+/-}$ mice regulating the NHEJ-mediated DNA repair and to stimulates DNA-PK activity in DSBs repair.

In accordance with this interesting function of TPO, I can confirm that NHEJ is the DNA-DSB repair pathway involved in the resolution of abscopal DNA damage and tumor response.

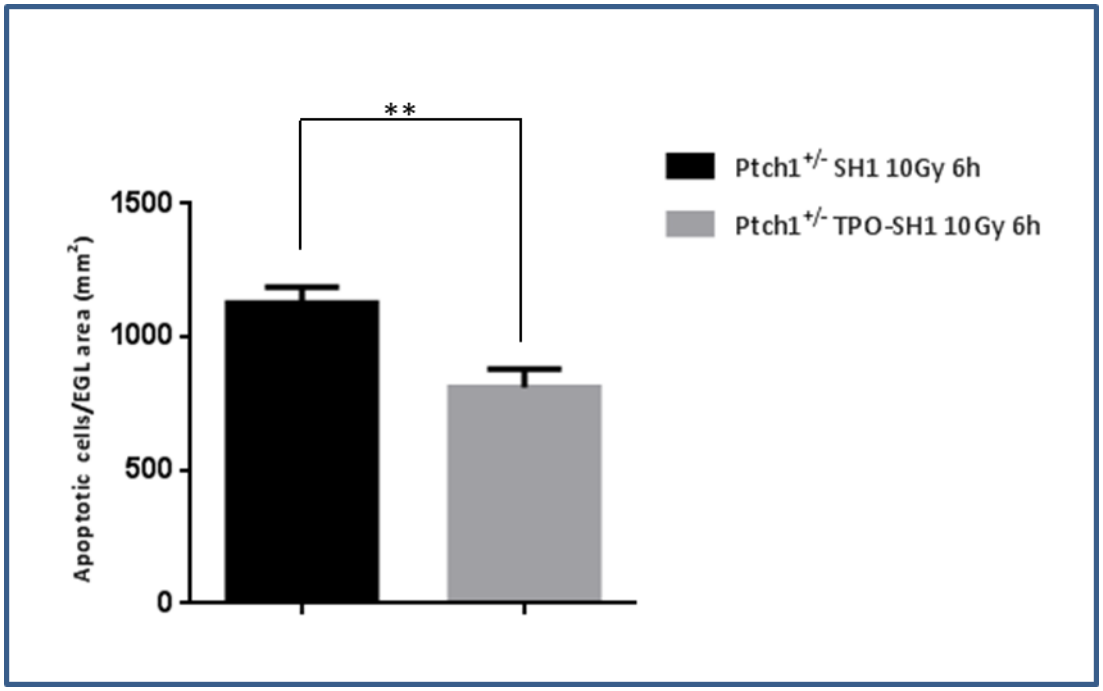

Figure 16. TPO induce significantly decrease (*P* value = 0.0017) in DNA damage (apoptotic index) in *Ptch1*$^{+/-}$ mice after SH1 irradiation. (**P value = 0.0017; Student's t-test).

DISCUSSION

Radiation is a key modality in the treatment of cancer and it is therefore of great practical interest to elucidate the mechanisms of *in vivo* non-targeted effects that can be modulated to enhance cancer cell killing, or lead to improved protection of normal tissues adjacent to cancerous lesions, including reduced risk of second malignancies.

The role of bystander signaling in increasing the efficacy of gene therapy approaches is well known and radiation-induced bystander responses may give new, additional opportunities to a more successful clinical approach[65].

In general, success or failure of standard clinical radiation treatment is determined by few crucial factors: repair of DNA damage, redistribution of cells in the cell cycle, repopulation, reoxygenation of hypoxic tumor areas[62], radio resistance of cancer stem cells (CSC). The CSC concept has been elevated to a higher level of significance in cancer therapy by recent evidence in several cancers that they can resist conventional treatments including ionizing radiation[70][71] and chemotherapy[72][73].

Concerning DNA damage repair, results from several groups have shown evidence of differential DNA damage responses in directly irradiated and bystander cells. In medium transfer experiments, Mothersill *et al*.[74] showed that repair-deficient human cell lines and surviving progeny underwent moderate to severe bystander-induced death effects compared with normal repair-proficient lines. Burdak-Rothkamm and colleagues found that inhibition of ataxia telangiectasia mutated (ATM) protein and DNA-PK could not suppress the induction of bystander γ-H2AX foci, whereas the mutation of ATM- and rad3-related (ATR) abrogated bystander foci induction[75]. Little *et al*.[64], by using mouse knockout cells lacking several genes in the non-homologous end joining (NHEJ) DNA repair pathway have shown the involvement of NHEJ in the bystander effect for chromosomal aberrations. Importantly, all these studies have provided initial evidence

that there may be differential DNA damage responses in directly-hit and bystander cells that could be exploited in future therapies.

In this study, I suggest that the interplay between abscopal damage response and DNA repair pathway status might play a critical role in the control of out-of-field tumour cells. To probe repair mechanisms relevant for bystander radiation effects I used the double knockout mice demonstrating that HR and NHEJ pathway express a different efficiency in processing abscopal damages and that the absence of NHEJ's components induce an increase of MB incidence.

At the first I analyzed the short term response demonstrating that after shielded irradiation *Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ mice are characterized by high apoptotic levels respect *Ptch1*$^{+/-}$*/RAD54*$^{-/-}$ mice. The primary response to DNA damage is the stimulation of DNA repair and the activation of cell cycle checkpoints. The biological goal of this primary response is to protect the damaged cell. Apoptosis is a secondary response to DNA damage, with the biological goal of protecting a multicellular organism against a damaged cell. One hallmark of cancer is intrinsic or acquired resistance to apoptosis. Surprisingly, recent studies demonstrate that CD95/Fas/Apo1 and p53 upregulated mediator of apoptosis/PUMA (potent inducers of the death receptor and the mitochondrial apoptotic pathways, respectively) promote tumorigenesis[76][77]. These findings provide important insights into the multifaceted roles of apoptosis in tumorigenesis suggesting apoptosis contributed to the high rate of cell loss in malignant tumors and, moreover, could promote tumor progression. In this context, my results support that the absence of the NHEJ pathway plays a critical role in the radiation abscopal response and that the apoptotic response can give additional information about tumor incidence.

MB incidence was evaluated by the characterization of the early development phase of MB. In fact, one remarkable feature of radio-induced medulloblastoma in *Ptch1*$^{+/-}$ mice is the development through microscopically recognizable preneoplastic lesions. Only a subset of PNLs eventually progress to medulloblastoma because a shift in the regression/progression balance in PNLs, evolution enhancing their propensity to develop into medulloblastoma, can occur. As demonstrating in other work the PNLs incidence represent a 'picture' of what would happen in a long-term carcinogenesis study. In this context, proving a link between preneoplastic lesions and tumors is critical if we are to base hypotheses about tumor progression on the study of these lesions.

We previously, in a transgenerational study[78], showed that, exploiting *Ptch1* heterozygous knockout mice, exposure of paternal germ cells to 1 Gy X-rays, at the spermatogonial stage, increased by a considerable 1.4-fold the offspring susceptibility to medulloblastoma induced by neonatal irradiation.

This effect gained further biological significance thanks to a number of supporting data on the immunohistochemically characterization of the target tissue and preneoplastic lesions (PNLs). These results altogether pointed to increased proliferation of cerebellar granule cell precursors and PNLs cells, which favored the development of frank tumours. The commitment to tumor progression strongly support the biological relevance of the 40% increase of cancer susceptibility obtained in the long-term carcinogenesis study.

Moreover, in another previous work[79], we focused to provide novel mechanistic insights into dose-, spatial- and time-dependent effects in abscopal signaling, used to detect the incidence of cerebellar microscopic tumors, the evaluation of early lesions/microtumors that represent bona fide neoplasms closely predicting final brain tumor incidence.

While, in most cases, the relationship between these lesions and the corresponding end-stage tumors has not been demonstrated directly, some evidences supporting the idea that studying the early stages of cancer can provide important insight into the molecular basis of the disease. Moreover, these studies indicate that PNLs represent a critical stage of tumorigenesis, during which cells have the capacity to decide whether to differentiate or whether to continue proliferating and give rise to medulloblastoma. Definitive evidence shows that PNLs give rise to tumors, and show that the predominant fate of PNLs that do not form tumors is differentiation[80].

Strong of these assumptions, after PCNA/NeuN analysis I can assume that the final incidence of MT, in *Ptch1*$^{+/-}$*/DNA-PKs*$^{+/-}$ mice will become 16/38 (42.10%) and that this value represent the final incidence of medulloblastoma.

This data supports the involvement of NHEJ pathway in the resolution of abscopal DNA damage and tumor induction.

To verify and confirm this hypothesis, I considered a pharmacological treatment strategy using a molecule capable of modulating the DNA repair pathway. After a bibliographic search[81], TPO has become my molecule of interest. I asked whether TPO could be a critical mediator sufficient to induce an *in vivo* modulation of DNA damage response in the cerebellum after partial-body irradiation. Hence, TPO was injected intraperitoneally P2 *Ptch1*$^{+/-}$ mice 30 min before radiation. I chose to test the TPO function in *Ptch1*$^{+/-}$ mice where the NHEJ is working just to allow the activity of the DNA repair pathway to be enhanced.

TPO and its receptor, Mpl, are primarily known for their role in megakaryopoiesis, but TPO has also been shown to support HSC quiescence during adult hematopoiesis, with the loss of signaling associated with bone marrow failure and thrombocytopenia[81].

Recently, in Cell Stem Cell, *De Lavel et al*. identified a novel role of TPO in the regulation of DNA repair in Hematopoietic Stem Cells (HSC)[68]. Exposure to genotoxic agents, such as ionizing radiation, induces DNA damage comprised of DSBs. DNA damage is repaired through two main pathways: HR and NHEJ. It's known that DNA repair is essential for cell survival, and studies have shown that NHEJ is necessary for HSC maintenance[82][83]. In this study, it was found that γH2AX foci, a marker of DSB formation, were significantly increased in Mpl-deficient HSCs and in their progenitors following IR exposure. Moreover, a TPO injection into mice prior to IR reduced the number of γH2AX foci in HSCs *in vivo*, while HSCs exposed to IR in the absence of TPO demonstrated an increased number of γH2AX foci.

Other experiments showed that TPO modulates the efficiency of the NHEJ pathway by increasing the phosphorylation of the DNA-PK catalytic subunit, a major enzyme involved in NHEJ. Pharmacological or genetic inhibition of DNA-PK abrogated TPO-mediated DNA repair[84]. Interestingly, the other cytokines involved in HSC maintenance and expansion, SCF and FLT3l, did not have the same effects as TPO, suggesting that DNA repair activity is a specific function of TPO. This is the first demonstration that a cytokine involved in HSC maintenance may also regulate DSB repair machinery.

Strong of these results, my study opens new prospects on the use of TPO. I show infact that TPO is able to decrease apoptosis in granule neuron precursors 6 h after treatment (TPO combined with SH1 irradiation).

Since TPO treatment prior to IR exposure reduces DNA damage, TPO or TPO agonists could potentially be given to patients prior to receiving chemotherapy to reduce the risk of developing oncogenic mutations. Thus, TPO might be suited for clinical applications involving protection from DNA-damaging agents.

Developing drugs aimed at modulating DNA DSB repair activity is likely to have a profound impact on the efficacy of radiation therapy. These observations have made targeting proteins in the DNA DSB repair pathways a popular approach for potential cancer treatments[85][86].

This therapeutic approach could have a detrimental effect on the therapeutic efficacy because DNA damage not only causes tumor development but could also battle cancers by impairing cancer growth and ultimately triggering the death of malignant cells. Defects in DNA and/or DNA repair can cause cancer as well as promote its growth. When mutations affect tumor suppressor genes or oncogenes, cell might transform into cancer cells. Therefore, DNA repair is essential for preventing tumor development.

However, once a cancer has developed, DNA damage can be exploited to reduce cancerous growth and evoke apoptotic demise of cancer cells. Thus, chemo- and radiotherapies are still today, over 60 years after having been first introduced into tumor therapy, important strategies to fight cancer. Given the central role of genome instability in triggering and treating cancer, it is likely that genotoxic treatments will remain an important avenue of cancer therapy. Also, the better understanding of DNA repair systems will allow therapies that specifically target selected repair pathways. It will be of particular importance to gain a deeper understanding how the various DNA repair systems interact with each other in the context of cellular homeostasis and DNA metabolism in order to optimize targeted approaches to cancer therapy[87].

FUTURE WORKS

Future works will be addressed to better understand some aspects.

ONGOING WORKS

It is very important to investigate the efficacy of TPO on the capacity to resolve direct DNA damage induced by radiation; in fact, it is known that the quality of the abscopal DNA damage and direct DNA damage could be different. The direct induction of the damage could produce a frequency of DSB in close proximity to one another (cluster), causing more complex damage more difficult to be repaired[88][89][90] than abscopal damage. To this aim, a new experimental set up is in progress and new samples and results will be collected and analyzed.

…FUTURE PROSPECTIVES

If TPO will be able to resolve the direct DNA damage with the same efficacy, as second step will be develop combined protocols of TPO treatment/irradiation in order to test benefit:risk ratio of this treatment plan. To this aim, I will establish murine MB allografts, using MB tumors from *Ptch1*$^{+/-}$ mice. Single cell suspensions from tumors will be injected in NOD/SCID mice with an optimized protocol[91] and the activity of the TPO, in combination with radiation-therapy treatment (therapeutic doses), will be tested by controlling the growth/regression of tumors.

CONCLUSIONS

The 2015 Nobel Prize in Chemistry was awarded to three DNA repair researchers Paul Modrich, Aziz Sancar and Thomas Lindahl, who detailed the molecular mechanisms of MMR, NER and BER, respectively. The advances in our understanding of these pathways have been instrumental in developing novel agents to block or, in some cases, enhance repair activity. The cadre of proteins and enzymes that respond to and repair DNA damage holds considerable potential to impact human health.

In this study, I highlighted recent advances in targeting DNA repair to pave the way for future DNA repair targeted agents and their use in cancer therapy especially in the protection of unexposed tissues after partial-body irradiation relevant in the radiotherapy context. Future research on this topic may help to minimize the 'collateral' risks to normal tissues remote from the tumor target, but future works will be necessary to in order to test benefit: risk ratio of this combined therapeutic plan.

This burgeoning field of research is replete with promise and challenge, as more intricacies of each repair pathway are discovered.

SUPPLEMENTARY CHAPTER

MATERIALS AND METHODS

ANIMAL TREATMENT AND IRRADIATION

Mice were housed under conventional conditions with food and water available ad libitum and a 12 hours light cycle.

Mice were partial-body irradiated with 10Gy of X-rays at postnatal day 2 (P2)[79]. Irradiation was performed using a Gilardoni CHF 320 G xray generator (Gilardoni, Mandello del Lario, Italy) operated at 250 kVp, 1 mA for 1 Gy, and 15 mA for 2, 3, and 10 Gy, with Half-Value Layer Z 1.6 mm Cu (additional filtration of 2.0 mm Al and 0.5 mm Cu). Additional groups of mice were left untreated.

Mice were irradiated with 4μm thick individual lead cylinder shields designed to protect approximately two thirds of the body whereas the hindmost part was directly exposed to radiation (SH1 shielded geometry, Fig. S1). Marked hair-growth delay at postnatal day 10 demarcates irradiated from shielded areas.

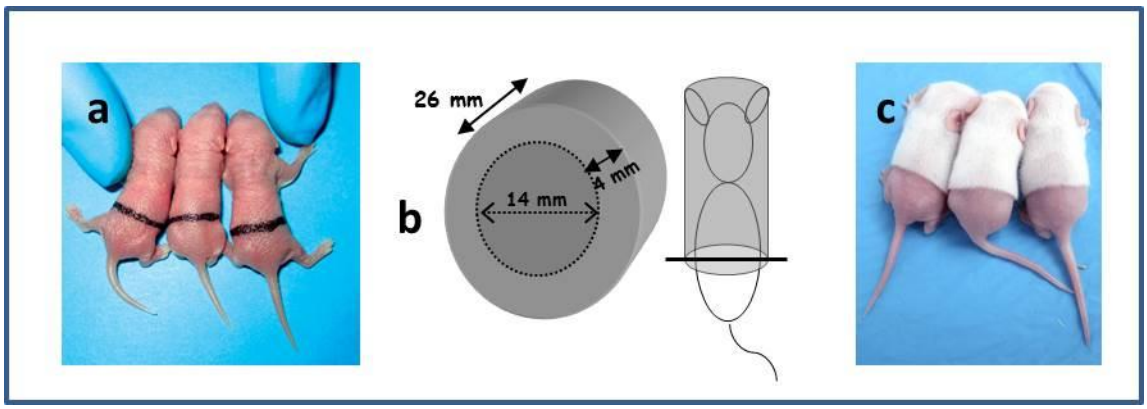

Figure S1. Irradiation set up for shielded irradiation. (a) Demarcation between exposed and shielded regions of neonatal (P2) *Ptch1*$^{+/-}$ mice; (b) Characteristics of the lead shields; (c) Demarcation between exposed and shielded regions at P10 due to hair-growth delay in exposed skin.

THROMBOPOIETIN INJECTION *IN VIVO*

Thrombopoietin (TPO) (Recombinant Murine TPO, PeproTech) (8 mg/kg body weight)[67] were intraperitoneal injected in newborn (P2) *Ptch1*$^{+/-}$ mice, 30 min before SH1 shielded irradiation with 10 Gy of X-rays. To evaluate the effect of TPO injection *in vivo* mice were sacrificed 6h post irradiation and analyzed for apoptotic response.
TPO was injected in *Ptch1*$^{+/-}$ mice in which both DNA repair machineries are working.

APOPTOTIC INDEX EVALUATION

To investigate the mechanisms of different susceptibility to radiation-induced MB tumorigenesis of mice cerebellum of different genotype, I examined the apoptotic response to shielded radiation-induced DNA damage in pups at P2.
Brains for each genotype (*Ptch1*$^{+/-}$*/DNA-PKcs*$^{-/-}$ and *Ptch1*$^{+/-}$*/Rad54*$^{-/-}$), were collected and fixed at 6 hours post-irradiation. Sections were cut at 4 μm thickness and stained with hematoxylin-and-eosin. EGL cells whit signs of nuclear chromatin condensation were counted. Apoptotic values were calculated as the percentage of pyknotic nuclei relative to the total EGL area.

HISTOLOGICAL ANALYSIS AND TUMOR QUANTIFICATION

Mice were observed daily for their lifespan. Upon decline of health (that is, severe weight loss, paralysis, ruffling of fur or inactivity), they were killed and autopsied. Normally appearing and tumor-bearing brains were fixed in 10% buffered formalin.

Samples were processed for histological analysis using standard methods. MB incidence was expressed as the percentage of mice with tumors.

In according with preneoplastic lesions development, described above, the incidence of preneoplastic cerebellar lesions was determined on histological sections of 8 weeks old asymptomatic mice. The incidence of cerebellar microscopic tumors was determined on hematoxylin-and-eosin stained of serial sections of the entire cerebellum, recovered with intervals of 100 μm, and expressed as the percentage of mice bearing microtumors[54].

Morphometric analysis to measure PNLs cross sectional areas was carried out using imaging software NIS-Elements BR 4.00.05 (Nikon Instruments Europe B.V., Italy).

The PNLs are classified in order of their dimensions: as nodules (area < 5×10^5 μm^2) or as microtumors (area > 5×10^5 μm^2).

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF PRENEOPLASTIC LESIONS

Brain sections were cut at 4 μm thickness for immunohistochemical analysis of PCNA (monoclonal 1:100; Millipore, Billerica, MA, USA) and NeuN (monoclonal 1:100; Millipore) performed using the HistoMouse ABCAM Kit, according to manufacturer's instructions. Immunohistochemical scoring was carried out by HistoQuest (TissueGnostics, Vienna, Austria) analysis software. Three frames for PNLs of *Ptc1*$^{+/-}$*/Rad54*$^{-/-}$ and *Ptc1*$^{+/-}$*/DNA-Pks*$^{+/-}$ SH1 irradiated mice, were captured by HistoFAXS software (TissueGnostics GmbH, Vienna, Austria) at 40x magnification. Specific regions of interest (PNLs) were analyzed with HistoQuest software (TissueGnostics) for automatic color separation and quantification. Expression levels were evaluated as percentage of positive (brown stained) stained area per mm^2.

The immunoistochemical analysis was carried out on small microlesions (area < 5×10^5 μm^2) to evaluate the balance between markers of proliferation/differentiation in order to assess their propensity to progress/regress into MB or to regress to normal tissue.

HISTOQUEST SOFTWARE

HistoQuest is a brightfield image analysis software for the FACS-like analysis of samples stained with immunohistochemical or histochemical stains. HistoQuest is a software that uses patented cell-identification algorithms for nuclear segmentation. The software programs are based on single cell detection by identification of nuclear structures. A sequence of mathematically realized processing steps applied to an image or part of an image, with the aim to extract specific information.

STATISTICS ANALYSIS

Analyses were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA). Apoptotic indexes are reported as means ±s.e., and the Student's t-test was used for determination of statistical difference between groups. Fisher's exact test is used for analysis of tumor incidence. P-value 00.05 was considered statistically significant.

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PUBLICATIONS

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